

PUBLIC TRANSLATION INTO ENGLISH-----

Descriptive Memory-----

Of the Invention Patent-----

On-----

Recombinant Human Erythropoietin Producing Cell Line and the Recombinant Human Erythropoietin produced by this cell line. -----

Applied for-----

Bio Sidus S. A. -----

For the term of 20 years. -----

There follows the logotype of BIO SIDUS. Constitución 4234 – 1254 Buenos Aires – Argentina. -----

Cell Line producing Recombinant Human Erythropoietin and the Recombinant Human Erythropoietin Produced by this Cell. -----

***I. Technical description of the invention***-----

The present invention relates to a recombinant human erythropoietin (EPO)-producing cell line, and to the EPO thus produced. Isolation and construction of a gene encoding for EPO, its cloning in proper plasmids to be transfected in mammalian cells. Selection of EPO-producing cell lines, its further culture and production of recombinant EPO. -----

***II. Technical field of the invention***-----

This invention refers to a recombinant EPO-producing cell line that includes a sequence encoding for EPO with only one promoter that regulates its expression. This invention is also referred to a method to produce EPO.-----

***III. Background of the invention***-----

EPO is a glycoprotein that stimulates erythroblast differentiation in the bone marrow, thus increasing the circulating blood erythrocyte count. The mean life of

erythrocytes in humans is 120 days and therefore, a human being losses 1/120 erythrocytes each day. This loss must be continuously restored to maintain a stable amount of red blood cells. -----

Existence of EPO was first postulated in the turn of the century and was definitely proved by Reissman and Erslev early in the '50s. See Carnot et al, *C.R. Acad. Sci.* (France), 143, 384-6 (1906); Carnot et al, *C.R. Acad. Sci.* (France), 143, 432-5 (1906); Carnot, et al., *C.R. Soc. Biol.*, 111, 344-6 (1906); Carnot, *C.R. Soc. Biol.*, 111, 463-5 (1906); Reissman, *Blood*, 1950, 5, 372-80 (1950) and Erslev, -----

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AR-98-01-05609 (Este número se repite al pie de cada una de las hojas del cuerpo de la patente, incluyéndose también al pie de cada una de las hojas de las Referencias Adicionales).-----

*Blood*, 8, 349-57 (1953). Reissman and Erslev's experiments were promptly confirmed by other researchers. See Hodgson et al, *Blood*, 9, 299-309 (1954); Gordon, et al., *Proc. Soc. Exp. Biol. Med.*, 86, 255-8 (1954) and Borsook, et al., *Blood*, 9, 734-42 (1954). -----

Identification of the producing site was an issue of deep debate. Successive experiments led to identify kidney as the main organ and peritubular interstitial cells as the synthesis site. See Jacobson, et al., *Nature*, 179, 633-4 (1957); Kuratowska, et al., *Blood*, 18, 527-34 (1961); Fisher, *Acta Hematol.*, 26, 224-32 (1961); Fisher, et al., *Nature*, 205, 611-2 (1965); Frenkel, et al., *Ann. N.Y. Acad.*

*Sci.*, 149, 1, 292-3 (1968); Busuttil, et al., *Proc. Soc. Exp. Biol. Med.*, 137, 1, 327-30 (1971); Busuttil, *Acta Haematol.*, (Switzerland), 47, 4, 238-42 (1972); Erslev, *Blood*, 44, 1, 77-85 (1974); Kazal, *Ann. Clin. Lab. Sci.*, 5, 2, 98-109 (1975); Sherwood, et al., *Endocrinology*, 99, 2, 504-10 (1976); Fisher, *Ann. Rev. Pharmacol. Toxicol.*, 28, 101-22 (1988); Jelkmann, et al., *Exp. Hematol.*, 11, 7, 581-8 (1983); Kurtz, et al., *Proc. Natl. Acad. Sci.*, (USA.), 80, 13, 4008-11 (1983); Caro, et al., *J. Lab. Clin. Med.*, 103, 6, 922-31 (1984); Caro, et al., *Exp. Hematol.*, 12, 357 (1984); Schuster, et al., *Blood*, 70, 1, 316-8 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Bondurant, et al., *Mol. Cell. Biol.*, 6, 7, 2731-3 (1986); Schuster, et al., *Blood*, 71, 2, 524-7 (1988); Koury, et al., *Blood*, 71, 2, 524-7 (1988); Lacombe, et al., *J. Clin. Invest.*, 81, 2, 620-3 (1988); Koury, et al., *Blood*, 74, 2, 645-51 (1989). -----

A smaller proportion, ranging from 10% through 15% of total EPO, is produced by the liver in adults. See Naughton, et al., *J. Surg. Oncol.*, 12, 3, 227-42 (1979); Liu, et al., *J. Surg. Oncol.*, 15, 2, 121-32 (1980); Dornfest, et al., *Ann. Clin. Lab. Sci.*, 11, 1, 37-46 (1981); Dinkelaar, et al., *Exp. Hematol.*, 9, 7, 796-803 (1981);

Caro, et al., *Am. J. Physiol.*, 244, 5 (1983); Dornfest, et al., *J. Lab. Clin. Med.*, 102, 2, 274-85 (1983); Naughton, et al., *Ann. Clin. Lab. Sci.*, 13, 5, 432-8 (1983); Jacobs, et al., *Nature*, 313, 6005, 806-10 (1985); Erslev, et al., *Med. Oncol. Tumor. Pharmacother.*, 3, 3-4, 159-64 (1986). EPO is proportionally produced to the degree of tissular hypoxia and its expression rises by increasing the number of producing cells. -----

EPO is a protein that has shown a great efficiency to treat anemia caused of different ethiology, specially anemia secondary to renal failure. Its therapeutical availability, however, was limited till recently due to the lack of a mass production method, since the quantity and quality of EPO obtained by any of the extractive systems knows were not sufficient. Recently, the use of recombinant DNA technology has made it possible to obtain large amounts of proteins. Application of these techniques to eukariotic cells has allowed large scale production of EPO. See patents U.S. 5.688.679 (Powell), 5.547.933 (Lin), 5.756.349 (Lin), 4.703.008 (Lin) and 4.677.195 (Hewick et al.). -----

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Recombinant DNA techniques are widely known and currently used. They are based on the manipulation of different genetic elements that facilitate, among other uses, ensamble and transfer genetic constructions and, consequently, allow production of recombinant proteins and the study of biological mechanisms. -----

See Frank-Kamenetskii, "Unraveling DNA" [Samaia Glavnaia Molekula] (Addison Wesley Longman Inc., Reading, Massachusetts, 1997); Brown, "Gene Cloning" (Chapman & Hall, London, England, 1995); Watson, et al., "Recombinant DNA", 2nd Ed. (Scientific American Books, New York, New York, 1992); Aberts et al., "Biología Molecular de la Célula" (Ediciones Omega, 1990); Innis et al., Eds., "PCR Protocols. A Guide to Methods and Applications" (Academic Press Inc., San Diego, California, 1990); Ehrlich, Ed., "PCR Technology. Principles and Applications for DNA Amplification" (Stockton Press, New York, New York, 1989); Sambrook et al., "Molecular Cloning. A Laboratory Manual" (Cold Spring Harbor Laboratory Press, 1989); Bishop et al., "Nucleic Acid and Protein Sequence. A Practical Approach" (IRL Press 1987); Reznikoff, Ed., "Maximizing Gene Expression" (Butterworths Publishers, Stoneham, Massachusetts, 1987); Davis et al., "Basic Methods in Molecular Biology" (Elsevier Science Publishing Co., New York, New York, 1986); Watson, "The Double Helix" (Penguin Books USA Inc., New York, New York, 1969) -----

There follows a rough description of the biological aspects on which recombinant DNA technology is based: -----

DNA (deoxyribonucleic acid) is the genetic material of all living cells and some viruses. Polymeric chains of four different nucleotides form the DNA, each of them being a purine or pyrimidine bound to a desoxyribose, in turn linked to a phosphate group. These four nucleotides are: adenine (A), cytosine (C), guanine (G) and thymine (T). -----

DNA chains are formed by linkages between nucleotides, where the phosphate in position 5' of the deoxyribose of one nucleotide is bound to the 3' position of the deoxyribose of the previous nucleotide. Synthesis *in vivo* occurs from 5' to 3', which is the conventional direction adopted to describe DNA sequences.

Functional DNA is presented as a double helix of complementary bases, where chains are held together by hydrogen bonds formed between A's and C's of one chain and T's and G's of the complementary chain, respectively. This is the reason why they are referred to as "base pairs". -----

The chains are also antiparallel, that is, the 5' end of each helix is matched to the 3' end of the other, as depicted below: -----

5' ---TACGTAC---3'-----

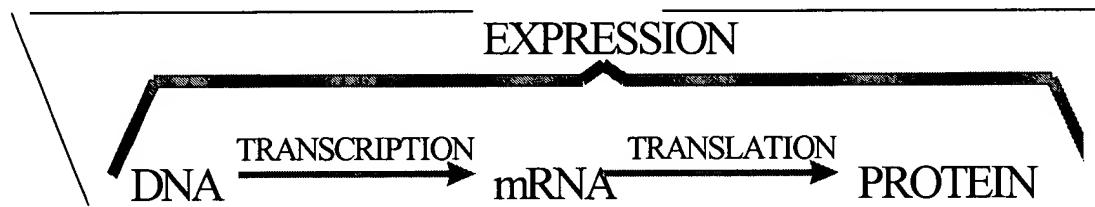
3' ---ATGCATC---5'-----

For protein synthesis to occur certain DNA coding regions are first transcribed to messenger RNA (mRNA). The mRNA is translated in turn into a protein. Each of the DNA coding regions for a protein is called a gene. -----

In the case of nucleated cells, the synthesized RNA may be processed in the nucleus (splicing) to result in mature RNA. This process is verified in bacteria as they have no nucleus. -----

Mature mRNA is then taken as a matrix to be translated into a protein, in a process in which transfer RNA (tRNA, small RNA chains carrying aminoacids and specifically alining them to form the protein) and ribosomes are the main participants.

Three mRNA bases (triplet or codon) code each amino acid. For instance, the AUG sequence in mRNA codes for the amino acid methionine. The nucleotides of each mRNA is thus translated into a specific aminoacid sequence, called "protein expression".



The amount of protein expressed depends, among other factors, on the presence of certain DNA regions called promoters, which affect the rate at which the expression process occurs per time unit.

In addition, there are DNA sequences that indicate the termination of transcription (terminators) and codons which indicate the end of translation (stop codons).

During the first years of the 1970 decade, "tools" (restriction enzymes, etc) and other techniques giving rise to recombinant DNA technology were developed.----  
At present, DNA technology involves the isolation of DNA fragments, either natural or synthetic, and their insertion into cells (i.e. bacteria, yeast, insect and mammalian cells) to render them capable of producing heterologous proteins such as EPO. The proteins obtained by this process are called recombinant proteins.

The application field of recombinant DNA technology is not limited to cultured cells, since genes can also be incorporated into multicellular organisms (i.e. plants, insects, mammals and fish). -----

The expression of heterologous proteins mainly requires the following elements:

1..A gene coding for the desired protein. The gene may be obtained using different techniques, such as: -----

A Isolation from genomic libraries. -----

B *In vitro* synthesis of DNA chains. There are commercially available equipments that synthesize relatively short DNA strands, making it possible to "make up" gene. -----

C.Amplification. Technology that allows to copy several times a DNA fragment of interest, such as a gene.-----

D.Others, i.e. as obtained from mRNA.-----

2.Poor promoters to express a protein in the cell of interest, in the desired amount and moment -----

3. Proper terminators so that transcription is correctly terminated.-----

4.Vectors. Genetic constructions that direct the gene with its promoter and terminator towards the inner region of the cell of interest incorporating the gene either in a chromosome or extrachromosomally. According to the system used, the incorporated gene may remain indefinitely in the cell and be transmitted to its progeny, or be lost in a relatively short term. There are multiple vector systems such as plasmids, and natural or modified viruses. It is also possible to use physical means of DNA introduction such as microinjection. Viruses and plasmids are obtained from nature and are genetically modified *in vitro* to achieve the desired characteristics.-----

5.Others. Additionally, other genetic elements may be necessary to improve the selection of cells receiving the gene (i.e. another gene conferring resistance to antibiotics) or to amplify the number of copies of the gene in each cell (genetic amplification).-----

An ideal expression system, should use vectors and genes being as simple as possible to minimize the risks of obtaining genetically unstable systems or wrong sequences resulting in undesired products. The use of simple vectors and genes reduces the time necessary to develop the system.-----

A fundamental consideration is that genetic simplicity should not disregard the productivity or quality of the protein produced.-----

To achieve the expression of the protein of interest, the appropriate corresponding gene is transfected with the proper vectors within the host cell

genetic material Transfection may be done by different techniques such as electroporation, precipitation with calcium phosphate and the use of liposomes.-- The gene of interest may be associated to other genes already known to confer resistance, for instance, to antibiotics such as geneticin, or to toxic agents such as methotrexate (MTX). This association allows the selection of the transfected cells in a stable manner, that is, those selected are capable of reproducing and transmitting the gene of interest to their progeny. Association also permits to select the recombinant cells showing the highest expression level.----- The recombinant product thus obtained is identified by analysis of its molecular weight, amino acid sequence, biological activity assays, etc..-----

To present the most widely used genetic engineering techniques to produce EPO are characterized as follow:

1. The use of EPO genes including fragments of neighbouring non-coding regions located at 5' and 3' of the same gene. It is conventionally believed that the presence of expression control elements located in said non-coding regions of the gene is necessary to achieve a high production of EPO. See patent US 5,688,679 .
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2. The employment of expression vectors with different promoters, based upon the premise that a combination of promoters induces a higher EPO production. Until now, the use of only one promoter included in the vector has resulted in a low level of protein expression. See patents US 4,703,008 (to Lin), US 4,677,195 and US 5,688,679. Average production of EPO using only one promoter is 200 µg/l/day. Maximum production of EPO reported using only one promoter is 10 mg/l/day.
3. The potential instability of the genetic systems and consequently of EPO production due to the complexity of the genetic constructions utilized.

#### ***IV. Detailed Description of the Invention***

The claimed invention consist in an eukariotic cell line that produces recombinant human EPO, obtained by means of transfection with an expression vector that includes a gene that encodes for human EPO, a unique promoter and a terminator as expression control elements. SEQ 1 identifies the EPO amino acid sequence that is codified by the gene used.

One of the advantages of this invention is that the EPO encoding gene used does not include fragments of the 5' and 3' neighbouring regions that do not encode for the protein. Despite this, the system claimed produces large amounts of EPO.

An additional advantage of this invention is the production of large amounts of

EPO using expression vectors bearing only one promoter. By using the claimed method, it is possible to obtain more than 50 mg of EPO per liter of culture per day, that is, over five-fold the EPO production level claimed by already known methods. -----

- Combination of the EPO-codifying gene used in this invention and a simple promoter showed, surprisingly, to operate efficiently, obtaining stably transfected cells that produce EPO yields comparable to, or even higher than, the ones reported using in theory more adequate genetic constructions, though more complex and harder to manipulate. -----

A further additional advantage of the claimed invention is cotransfection with two

vectors that confer different resistances, thus making selection, genetic amplification and maintenance of co-transfected producing cells easier.-----

In order to obtain the cell line subject of the claimed invention, genomic DNA extracted from human white blood cells is prepared. The EPO-encoding gene is obtained from conditioned DNA. To achieve this, the gene is amplified using primers that avoid presence of 5' and 3' neighbouring regions of the EPO gene that do not encode for the protein. These primers include in their 5' end region, restriction sites that remain then inserted to both ends of the isolated gene and thus facilitate further cloning. -----

Then, the amplified gene is cloned in a bacterial vector and is sequenced. Once it has been proved that the sequence obtained is correct, the gene is cloned in the Xho I-Hind III sites of an expression vector for eukaryotic cells that employs, as expression controller, only the early promoter of SV40 and its terminator. The vector confers resistance to Geneticin and Ampicillin.-----

Then, CHO cells are cotransfected with the expression vector obtained and a second vector that confers resistance to Methotrexate.-----

Stably transfected cells are selected according to their resistance to Geneticin and EPO expression is amplified by selecting cells resistant to increasing amounts of Methotrexate. -----

Finally, clones are selected according to their productivity level as measured by radioimmunoassay. Culture supernatants of the most productive clones are used to test the identity of the EPO produced and its biological activity by SDS-PAGE, Western blot, glycanase treatment followed by SDS-PAGE, isoelectrofocusing, complete protein sequence and in vivo biological activity in ex-hypoxic polycythemic mice challenged versus the international EPO standard.

Processes listed above are performed employing molecular biology techniques that are exemplified below: -----

***Example 1 Preparation of Human Genomic DNA.***-----

10 ml of blood in 10 mM EDTA ( pH 8) were extracted from a clinically healthy adult male subject. The blood was transferred in 5 ml aliquots to two 50 ml tubes, to which 45 ml of a solution containing 0.3 M of saccharose, 10 mM Tris-HCL (pH 7.5), 5 mM Mg Cl<sub>2</sub> and 1% of Triton X 100 was added. The resulting solution was stored at 4° C.

The solution was then placed on ice for 10 minutes and centrifuged for 10 minutes at 1000 g and at 4°C. The supernatant was discarded and the pellet was washed up several times with a 0.075 M NaCl solution containing 0.025 M EDTA (pH 8), followed by centrifugation for 10 minutes at 1000 g and at 4°C.

The resulting pellet thus obtained was resuspended in 3 ml of a 10mM Tris-HCl (pH 8), 400 mM NaCl, 2 mM EDTA (pH 8) solution. 200 µl of 10 % SDS (sodium dodecyl sulphate) and 500 µl K proteinase (1 mg/ml in 1 % SDS and 2 mM EDTA pH 8) were then added, and the solution was incubated overnight at 37°C.

After this incubation, 1 ml of NaCl saturated solution was added; the solution was shaken and then centrifuged at 2500 g for 15 minutes.

The supernatant was transferred to a 15 ml tube where the volume was duplicated by the addition of isopropanol. The supernatant and isopropanol were gently mixed by inversion of the tube and stored at room temperature until a DNA precipitate was formed, which was "fished" with a Pasteur blent glass pipette.

The DNA was placed in a 2 mL tube, and 1 mL of 70 % ethanol was added. After the solution was left stand for one minute, the supernatant was discarded and the precipitate was left to dry. After drying, the precipitate was suspended in 500 µl of TE (10 mM Tris-HCl pH 8 - 1 mM EDTA).

Concentration of DNA solution was calculated by measuring the absorbance at

260 nm of a 1:1000 dilution of this solution. Each unit of optical density was considered to have 50 µg of genomic DNA. Once the concentration was known, a solution was prepared with 500 ng of genomic DNA per µl in TE.

***Example 2 . Isolation of EPO encoding gene***

Gene encoding for EPO was prepared from 500 ng of human genomic DNA obtained in Example 1, adding 400 ng of each of the EPO 1 and EPO 2 primers, in an aqueous 2.5 mM solution containing each deoxynucleotide (dATP, dCTP, dGTP and dTTP), 2.5 units of Taq DNA polymerase (Perkin Elmer) in a final volume of 100 µl using the buffer recommended by the manufacturer. A Perkin Elmer-Cetus Thermal Cycler 480 was used and was programmed for 30 cycles of: 1 minute at 93°C, 1 minute at 55°C and 3 minutes at 72°C. From this reaction, a fragment of approximately 2170 pair bases containing the EPO gene was obtained.

Sequence of olygonucleotides employed was:

EPO 1: 5' GAATTCTCGAGATGGGGTGACGGTGAG 3' (SEQ NO:2), corresponding to the first bases translated from the EPO gene with the addition of a recognition site for the Xho I enzyme and one for the recognition of Eco RI enzyme to the 5' end. These sites were used in the subsequent cloning steps.

EPO 2: 5' AAGCTTGGACACACCTGGTCATCTG 3' (SEQ NO:3), complementary to the last translated bases and to some of the non-encoding 3' of the EPO gene. A site for the recognition of the Hind III enzyme was added to-

the 3' end. This site was used in subsequent cloning steps. ---

The sequence obtained is as follows (SEQ NO:4):

ctgggagcccaggtaggtggagcggacactctgcttgccttcgttaagaaggggagaagggtctgctaa  
ggagtacaggaactgtccgtattccctcccttctgtggcactgcagcgacccctgtttccctggcagaaggaa  
gccatctcccccccaagatgcggcctcagctgctccactccgaacaatcactgctgacacttccgcaaactttcc  
gagtctactccaattccctccggggaaagctgaagctgtacacaggggaggcctgcaggacaggggacagat  
gaccaggtgtccaagc**t**

The first translated atg codon, as well as the tga “stop” codon are underlined. The sequences of restriction sites utilized in the cloning are shown in bold italics.

**Example 3. Cloning and sequencing of the Isolated Gene.**

A fragment of approximately 2170 base pairs corresponding to the EPO gene was purified and the ends were blunted by treatment with the RNA polymerase Klenow fragment and cloned in the Sma I site of a M13mp18 vector, following standard techniques applied in molecular biology. The recombinant plasmids obtained were cut with the Xho I and Hind III enzymes; the presence of the insert was verified by electrophoresis of the product resulting from the restriction fragments in a 0.8 % agarose gel developed with ethyldium bromide stain. A positive clone (two bands, one having approximately 2200 base pairs and the other one corresponding to the linear vector) was chosen and manually sequenced according to the Sanger’s technique using a “T7 sequencing kit” (Pharmacia) and with the aid of an automatic sequences Model 370 A Applied Biosystems International. For each sequencing system the protocols recommended by the manufacturers were followed.

**Example 4. Vectors for Eukaryotic Cells**

**1. Construction of pVex 1 Vector**

The pVex1 vector was built following the conventional techniques used in molecular biology. It consisted of:

- a. Fragments of the bacteria1 pBR322 vector, which conferred a bacterial replication origin and resistance to ampicillin, for amplification and selection of the vector in *E. coli*.

b. Immediately close to 3' of a) an early promoter of the SV40 virus is located, which allows the expression of the genes cloned at 5' from this element.-----  
c.Immediately close to 3' of b) the Xho I and Hind III cloning sites are located, which allow insertion of the genes to be expressed.-----  
d.Immediately close to 3' of c) the polyadenylation signal of the SV40 virus is located, which allows the proper polyadenylation of the specific transcripts of the gene cloned in c). -----  
e.Immediately close to 3' of d) the TK promoter and the gene coding for neomycin phosphotransferase plus the polyadenylation signal are located to allow the selection of stably transfected cells through selection by resistance to neomycin and neomycin-derived antibiotics such as genetycin. The 3' end of e) is linked to the 5' end of a). -----

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A specimen of vector pVex 1 is deposited in the Argentine Cell Bank Association, deposit number ABAC-P-001.-----

**B. pDHFR Vector-----**

The pDHFR vector confers resistance to ampicillin to aid in selection in bacteria. and includes the DNA copy encoding for mice dehydrofolate reductase (DHFR), whose expression is controlled by the SV40 virus early promoter and its polyadenylation signal. -----

The coexpression of DHFR and the EPO-encoding gene allows, through selection by adding methotrexate (MTX) to the culture medium, several times-amplification of EPO expression achieved with the pVex 1-EPO vector.-----

A specimen of the pDHFR vector is deposited in the Argentine Cell Bank Association, deposit number ABAC-P-002. -----

***Example 5. Cloning of the EPO-Encoding gene into an Expression Vector--***

The sequenced gene was removed by cleavage with the Xho I-Hind III enzymes

of the vector where it was cloned in Example 3. It was then isolated and cloned in the same restriction sites of the pVex I vector. A positive pVex-EPO clone was isolated. All these operations were performed according to the conventional genetic engineering techniques. See Brown, *Gene Cloning* Chapman & Hall, London, England, 1995; Watson, et al., *Recombinant DNA*, 2<sup>nd</sup> Ed. Scientific American Books, New York, New York, 1992; Sambrook et al, *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989; Bishop et al., *Nucleic Acid and Protein Sequence. A Practical Approach*, IRL Press, 1987; Davis et al., *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., New York, New York, 1986.

**Example 6. Co-transfection and amplification with MTX-----**

A CHO (Chinese Hamster Ovary) cell line, mutated to be deficient in the DHFR-enzyme gene (CHO-DHFR), was used to facilitate the genetic amplification with MTX.

During this whole process, cells were grown at 37°C in a 5% of CO<sub>2</sub> atmosphere.

CHO cells were cotransfected following the calcium phosphate technique which, for a 90 mm-diameter Petri dish, consists in:

- (a) Replacing the culture medium (alpha-MEM, with 10 % of bovine fetal serum) with fresh medium 4-8 hours before transfection.
- (b) Adding to a 5 mL tube a 10 g/l HEPES solution (pH 7.1), 16 g/l NaCl and 10 µl of a 35 mM Na<sub>2</sub>HPO<sub>4</sub> and 35 mM of NaH<sub>2</sub>PO<sub>4</sub> solution.
- (c) Preparing in a separate 1.5 ml tube a solution with 60 µl of 2 M CaCl<sub>2</sub> and 10 µg of each DNA to be transfected (pVex-EPO and pDHFR). Water was added until the volume reached 500 µl. The pDHFR plasmid described in Example b is based on the pBR 322 plasmid, which confers resistance to ampicillin, can replicate in *E.coli*, has the DHFR gene cloned between the early promoter and

the terminator of the SV40, and allows the expression of the DHFR protein in CHO cells. This protein confers resistance to methotrexate, which can be then used to select cells having a high erythropoietin productivity.-----

(d) Adding drop by drop, the solution containing DNA and CaCl<sub>2</sub> to the tube containing Hepes, while air is bubbled to obtain a rapid mixing and to make the local concentrations as small as possible. This method facilitates the formation of a very thin precipitate which is more effectively incorporated by the cells.-----

(e) The solution is allowed to settle for 30 minutes and then is poured on the cells. -----

(f) The solution is distributed among the cells through gentle shaking, and left overnight in an incubator at 37° C under a 5% CO<sub>2</sub> atmosphere.-----

(g) The cells are washed twice with PBS (8 g NaCl; 0.2 g KCl; 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g NaH<sub>2</sub>PO<sub>4</sub>, water is added until the volume reached 1 liter and pH is adjusted to 7.4 with HCl. Fresh culture medium is then added.-----

Twenty four hours after transfection, the selection with genetycin (G 418) at a final concentration of 600 µg/ml was begun. The cells that stably incorporated the pVex-EPO plasmid were able to resist the antibiotic while all the others died after 25 days. Resistant colonies were selected and their productivity was assayed. Once the clones were identified, the three most productive ones were selected. -----

Taking advantage of the genetic constructions used in the invention, a selection was performed with each of the three clones using a second selective agent: MTX at different concentrations: 10<sup>-8</sup>M, 10<sup>-7</sup>M, 10<sup>-6</sup>M, 10<sup>-5</sup>M. For that purpose, the culture medium was changed to alpha-MEM without nucleosides, supplemented with 10% dialyzed bovine fetal serum. It was essential to perform the dialysis process according to the following schedule: for 100 ml of serum, the serum is placed in a dialysis bag with a porosity under 3000 Da (with a higher porosity, growth factors would be lost, and the cells would not be able to grow

and reproduce), the bag is hermetically closed, and completely immersed in a recipient with 5 liters of bidistilled water; where it is left at 4°C for 12 hours. After this, the water is discarded and again 5 liters are added and the bag is then left stand at 4°C for an additional 12 hour period. Then the dialysis bag is removed and the serum is recovered. Dialysis during shorter periods or with smaller volumes, or without replacing the water, would be worthless since a small amount of nucleotides could be left in serum, and therefore the selection with MTX would not work. Dialysis during longer periods would also be worthless since some proteins, necessary for cell growth, could precipitate and be lost.-----

***Example 7. Isolation of high productivity clones***-----

Clones that grew in  $10^{-7}$  and  $10^{-6}$  M of MTX were isolated, amplified in fresh alpha-MEM without nucleosides supplemented with 10% of dialyzed bovine fetal serum. Once grown, the culture supernatant was assayed to measure EPO production and secretion. For that purpose, a specific immunoassay was used.-- The process described above concluded with the selection of a clone of recombinant cells producing 50,000 µg of erythropoietin/liter of culture medium per day.-----

A specimen of recombinant cells employed is deposited in the Argentine Cell Bank Association, deposit number ABAC-L-200. -----

Cell transcripts specific for EPO were controlled as described in Example 8, to verify that there were no mistakes in the DNA sequence used or in its transcription. In order to identify the protein obtained, it was proceeded as described in Example 9. -----

***Example 8. Verification of the Specific messenger RNA Sequence Produced by the Recombinant Cells***-----

***A.Preparation of RNA from cells.***-----

Total RNA was prepared from producing cell lines, according to the following protocol: -----

a. 90-mm diameter Petri dish with confluent cells was washed twice with 10 ml of PBS.

b. Two ml of GTC buffer were then added and spread all over the dish. The GTC buffer was composed of: (1) 50 g guanidinium thiocyanate; (2) 0.5 g N-Lauroilsarcosin; (3) 2.5 ml 1 M sodium citrate, pH 7; (4) 0.7 ml  $\beta$ -mercaptoethanol; (5) 0.33 ml 30% antifoam agent (SIGMA); (6) H<sub>2</sub>O q.s.. 100 ml, pH 7.0.

Cells were lysed resulting in a highly viscous solution. The solution was transferred to a 15 ml tube, and the process above described was repeated once more using 2 ml of GTC buffer.

a) The 15 ml tube was vigorously shaken for 1 minute to break the DNA. Fractioning in a cesium chloride gradient was then performed. For that purpose, 4 ml of a solution containing CsCl (95.97 g CsCl and 2.5 ml of 1 M Sodium Acetate, pH 5.4, water was added to reach a volume of 100 ml) were poured in an ultracentrifuge tube. Over this solution and without mixing, the suspension of the cells in GTC was added. The tube was then filled with GTC buffer and ultracentrifuged at 20°C, for 20 hours at 31000 rpm.

b) The RNA remained at the bottom of the tube (pellet) and the DNA formed a band in the middle of the cesium chloride gradient.

c) The supernatant was discarded, taking special care to eliminate all of the DNA. The RNA-containing pellet was left to dry for 5 minutes.

d) The pellet was dissolved in 200 ml of water and transferred to a 1.5 ml tube.

e) 200 ml of 0.4 M Sodium Acetate, pH 4.8, and 2 volumes of ethanol were then added, the resulting solution was thoroughly mixed and left to stand for 30 minutes at -80°C.

f) The solution was then centrifuged in a microcentrifuge at 14000 rpm for 15 minutes, the supernatant was discarded and the precipitate was rinsed with 1 ml of 80 % ethanol.

g) The pellet was dried and redissolved in 100 ml of water. -----

f) The concentration of a 1:100 dilution of the RNA solution was measured at 260 nm (one optical density unit is equivalent to 40 mg of RNA). -----

NOTE: All the solutions and elements used were RNAase-free.-----

***b. Preparation of specific cDNA***-----

Specific cDNA was prepared following the directions of a kit intended for that purpose (cDNA Synthesis System Plus, Amersham - cat. RPN 1256). EPO2 oligonucleotide was used as specific the primer. -----

***c. Cloning of cDNA Encoding for EPO***-----

1/20th of the obtained cDNA was amplified using 400 ng of each the EPO2 and EPO3 oligonucleotides, and 2.5 mM of each deoxynucleotide in the proper buffer, and 2.5 units of Taq DNA polymerase, in a total volume of 100 ml. -----

Thirty five amplification cycles were performed as follows: 1 minute at 93°C, 1 minute at 55°C and 1 minute at 72°C. -----

EPO 3 was synthesized as described for EPO 1 and EPO-2, and its sequence (5' GAATTCCATGGGGTGCACGAATGTCC 3') (SEQ ID NO:5) corresponded to the first 20 bases encoding for the EPO cDNA, adding one site for the recognition of the Eco RI enzyme, to facilitate the subsequent genetic manipulations. -----

A fragment of approximately 600 base pairs was obtained, which was cloned in M13mp18 and M13mp19 vectors. -----

The presence of the insert in the clones with restriction fragments was assayed and sequenced in both directions to obtain the complete sequence, using the Sanger's method. -----

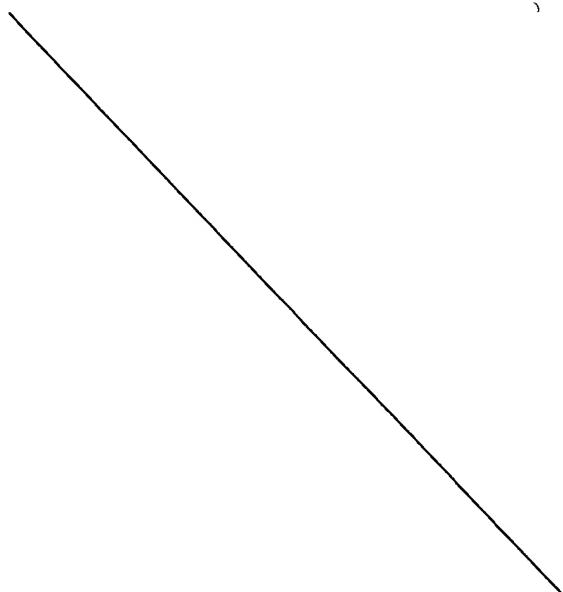
Due to the very high autocomplementarity of some regions of the gene, which gives rise to many and very ambiguous compressions in the radioautography, a sequencing kit using Taq DNA polymerase and modified bases was used. Lower

quality results were obtained, but the compressions were eliminated. The kit used was the Pharmacia-LKB Biotechnology *Gene aTaq*.-----  
The complete sequence of the human erythropoietin DNA copy, isolated and cloned, showed to encode for EPO. Therefore, no mistakes in the gene or in its transcription were possible.-----

***Example 9. Analysis on the EPO Produced***-----

The EPO obtained by culturing the cells of this example underwent different quality and identification assays. -----

- a) In a denaturing SDS-PAGE gel the EPO was identified as a wide band of molecular weight superior to 30 kDa. See Figure 1.-----
- b) The band was recognized by a monoclonal antibody and by a polyclonal antibody to human EPO in a "Western blot" assay. See Figure 2.-----
- c) Treatment with glycanases proved the existence of the glycosidic chains whose quantity and molecular weight were as expected. See Figure 3.-----
- d) The EPO produced proved to be composed of a series of species with isoelectric points ranging from 3.0 to 4.5. See Figure 4.-----
- e) The complete amino acid sequence of the isolated protein, purified from the culture supernatant of transfected cell lines showed total homology with natural human erythropoietin whose 165 aminoacid sequence is as follows (SEQ NO:1):



NH <sub>2</sub> -----	Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp	Ser	Arg
	Val	Leu	Glu	Arg	Tyr	Leu	Leu	Glu	Ala	Lys
	Glu	Ala	Glu	<u>Asn</u>	Ile	Thr	Thr	Gly	Cys	Ala
	Glu	Hys	Cys	Ser	Leu	Asn	Glu	<u>Asn</u>	Ile	Thr
	Val	Pro	Asp	Thr	Lys	Val	Asn	Phe	Tyr	Ala
	Trp	Lys	Arg	Met	Glu	Val	Gly	Gln	Gln	Ala
	Val	Glu	Val	Trp	Gln	Gly	Leu	Ala	Leu	Leu
	Ser	Glu	Ala	Val	Leu	Arg	Gly	Gln	Ala	Leu
	Leu	Val	<u>Asn</u>	Ser	Ser	Gln	Pro	Trp	Glu	Pro
	Leu	Gln	Leu	Hys	Val	Asp	Lys	Ala	Val	Ser
	Gly	Leu	Arg	Ser	Leu	Thr	Thr	Leu	Leu	Arg
	Ala	Leu	Gly	Ala	Gln	Lys	Glu	Ala	Ile	Ser
	Pro	Pro	Asp	Ala	Ala	<u>Ser</u>	Ala	Ala	Pro	Leu
	Arg	Thr	Ile	Thr	Ala	Asp	Thr	Phe	Arg	Lys

Gly	Lys	Leu	Lys	Leu	Tyr	Thr	Gly	Glu	Ala
Sys	Arg	Thr	Gly	Asp	Arg	-----	COOH	-----	

X Glycosylation sites-----

f) The presence of the four glycosylation sites on the 165 amino acid chain, as well as the complex carbohydrate structure, specifically, the sialic acid terminal residues, were demonstrated, as well as its correct *in vivo* biological activity, when assayed by the model of the ex-hypoxic polycythemic mouse assay, which showed a total parallelism versus the corresponding international standard.-----  
g) Productivity achieved, measured by a specific immunoassay, was 50 mg per liter of culture per day.-----

**V. Description of Diagrams-----**

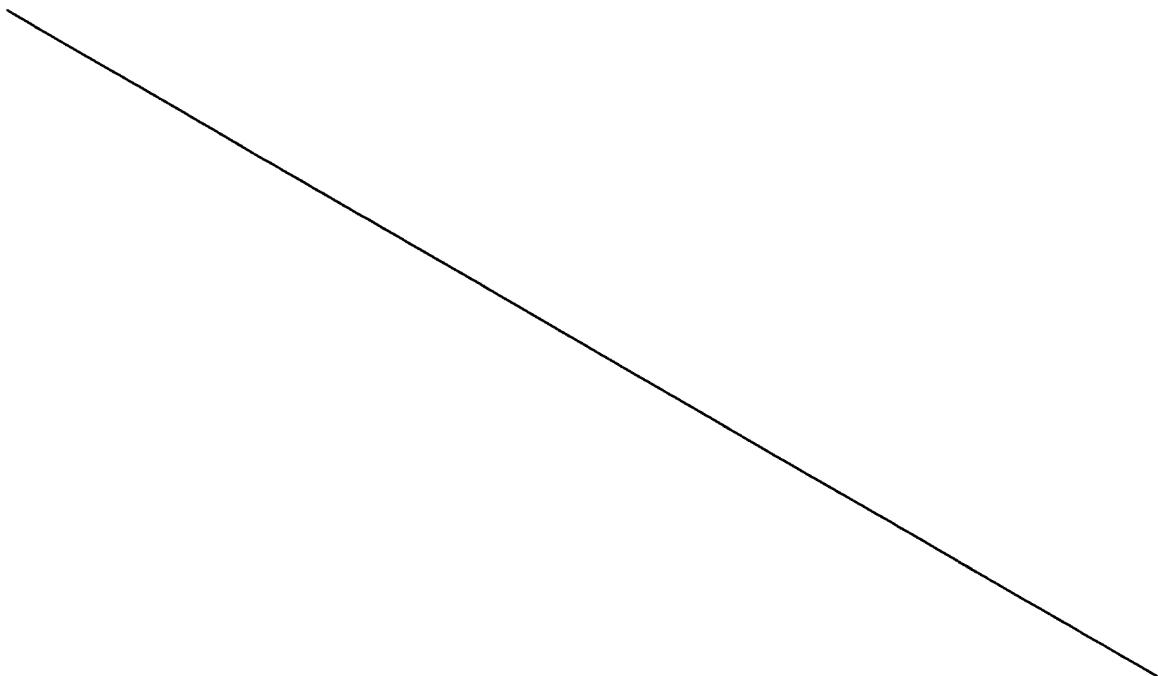
Figure 1 illustrates polyacrilamide gel (SDS-PAGE) analysis of an EPO sample obtained following the method described. In lanes 1, 4 and 7, molecular weight markers were loaded. In lanes 2, 3, 5 and 6, different masses of pure EPO obtained according to the claimed procedure were run. The purity of the product obtained and the apparent molecular weight slightly exceeding 30 kDa - coincident with the one reported for urinary human EPO, can be clearly observed. Figure 2 illustrates a Western blot analysis of an EPO sample obtained according to the method described. Identity of the EPO produced is assessed, since it is recognized by an antibody to human EPO. In lane 1, a human EPO standard was loaded, in lane 2, molecular weight markers and in lanes 3 through 5, EPO samples obtained according to the claimed method.-----

Figure 3 shows a SDS-PAGE analysis of a pure EPO sample obtained according to the method described, treated with glycanases. Molecular weight markers were loaded in lanes 1, 4 and 8. Lanes 2 and 7 correspond to untreated EPO. In lane 3, O-glycanase treated EPO was loaded; the presence of an O-

glycosylation is verified. In lane 5, N-glycanase partially degraded EPO was loaded. The presence of 3 N-glycosylations with the molecular weights expected for EPO can be verified. Lane 6 was loaded with EPO degraded with O-glycanase and N-glycanase, and the expected molecular weight for the fully deglycosylated protein was obtained.

---

Figure 4 illustrates a survey of the isoelectric points in pure EPO samples produced according to the method described. EPO samples were run in lanes 2, 3 and 4, isoelectric point markers in lanes 1 and 5. The presence of forms corresponding to EPO are verified, in an isoelectric point range of 3.0 to 4.5.



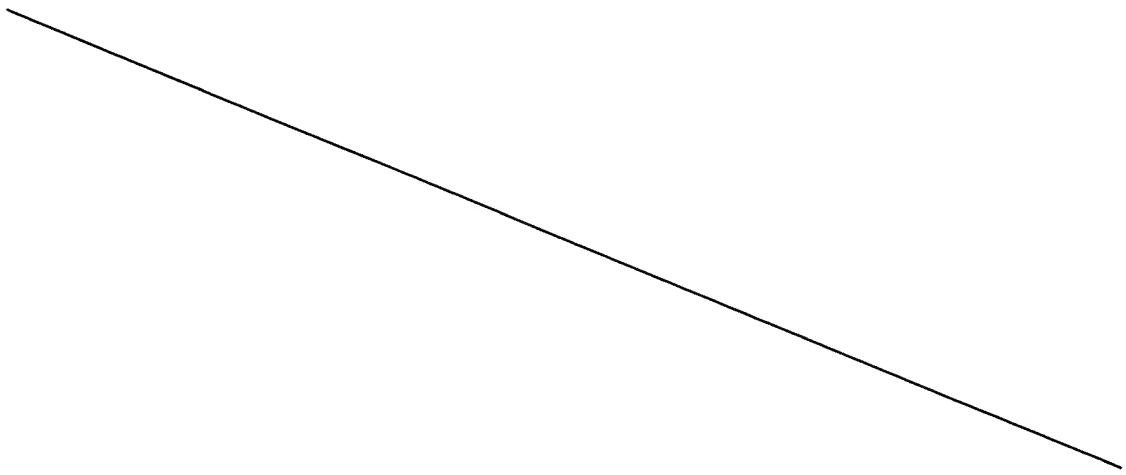
**VI. What Is Claimed Is:** -----

Having described and exemplified the nature and main subject of this invention, as well as the manner in which it can be operated, it is hereby stated to claim as of exclusive property and rights:-----

1. A CELL LINE PRODUCING RECOMBINANT HUMAN ERYTHROPOIETIN characterized by the following: a) the EPO encoding gene is the one detailed in SEQ 4 and consists only in the human EPO gene encoding region, without including 5' and 3'neighbouring regions of the EPO gene that do not encode for the protein, b) the genetic constructs used have, as control elements of EPO expression, a unique viral promoter and terminator, c) the method to construct the EPO encoding gene uses human DNA as source material. -----
2. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because the expression genetic systems conist in two vectors that have as control elements of the EPO exprssion only the early promoter of the SV40 virus and its terminator. -----
3. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because the genetic exprssion systems consist in two vectors that have as a control element for EPO expression the early promoter of the SV40 virus.-----
4. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because it comprises a pDHFR vector. -----
5. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because cell lines are selected using a double system, 1) resistance to geneticin and 2) resistant to increasing quantities of Methotrexate.-----

A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because the EPO thus obtained consists in

165 amino acids according to the following sequence: -----



NH <sub>2</sub> ---	Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp
Ser	Arg	Val	Leu	Glu	Arg	Tyr	Leu	
Leu	Glu	Ala	Lys	Glu	Ala	Glu	<u>Asn</u>	
Ile	Thr	Thr	Gly	Cys	Ala	Glu	Hys	
Cys	Ser	Leu	Asn	Glu	<u>Asn</u>	Ile	Thr	
Val	Pro	Asp	Thr	Lys	Val	Asn	Phe	
Tyr	Ala	Trp	Lys	Arg	Met	Glu	Val	
Gly	Gln	Gln	Ala	Val	Glu	Val	Trp	
Gln	Gly	Leu	Ala	Leu	Leu	Ser	Glu	
Ala	Val	Leu	Arg	Gly	Gln	Ala	Leu	
Leu	Val	<u>Asn</u>	Ser	Ser	Gln	Pro	Trp	
Glu	Pro	Leu	Gln	Leu	Hys	Val	Asp	
Lys	Ala	Val	Ser	Gly	Leu	Arg	Ser	
Leu	Thr	Thr	Leu	Leu	Arg	Ala	Leu	
Gly	Ala	Gln	Lys	Glu	Ala	Ile	Ser	
Pro	Pro	Asp	Ala	Ala	<u>Ser</u>	Ala	Ala	
Pro	Leu	Arg	Thr	Ile	Thr	Ala	Asp	
Thr	Phe	Arg	Lys	Leu	Phe	Arg	Val	
Tyr	Ser	Asn	Phe	Leu	Arg	Gly	Lys	
Leu	Lys	Leu	Tyr	Thr	Gly	Glu	Ala	
Cys	Arg	Thr	Gly	Asp---COOH	-----			

X Glycosylation sites -----

7. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because it surprisingly produces large amounts of EPO, higher than 50 mg/liter of culture medium/day.

8. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because cells are mammalian cells. -----

9. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because cells are CHO, COS, BHK, Namalwa, HeLa, Hep3B, Hep-G2 or other mammalian cells. -----

10. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because the cells are

CHO or COS.-----

11. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because the cells are CHO cells.-----

12. A CELL LINE THAT PRODUCES RECOMBINANT HUMAN ERYTHROPOIETIN according to claim 1, characterized because the genomic DNA used is obtained from human white blood cells.-----

There follows an illegible signature followed by a seal that reads: HUMBERTO M. DE PASQUALE. ATTORNEY.-----

**VII Diagrams**

Fig. 1. Polyacrilamide gel electrophoresis analysis (SDS-PAGE)

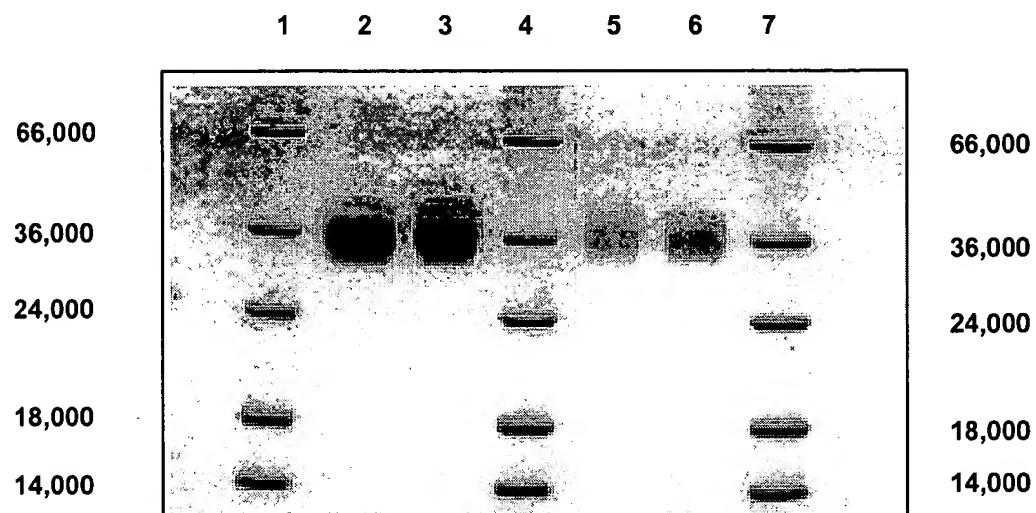


Fig. 2. Western blot analysis

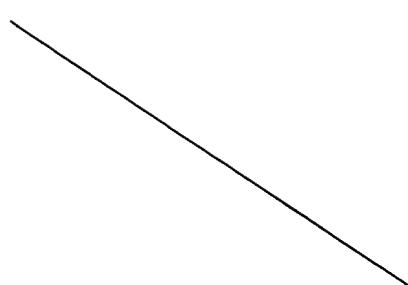
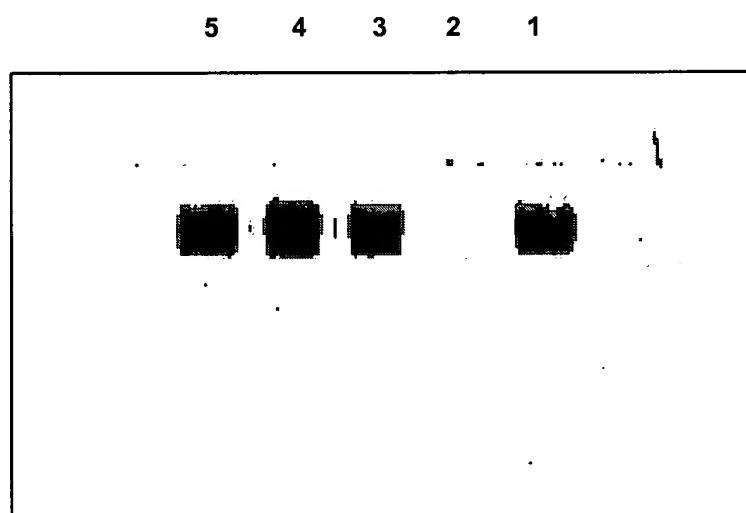


Fig. 3. SDS-PAGE analysis of EPO digestion with glycanases -----

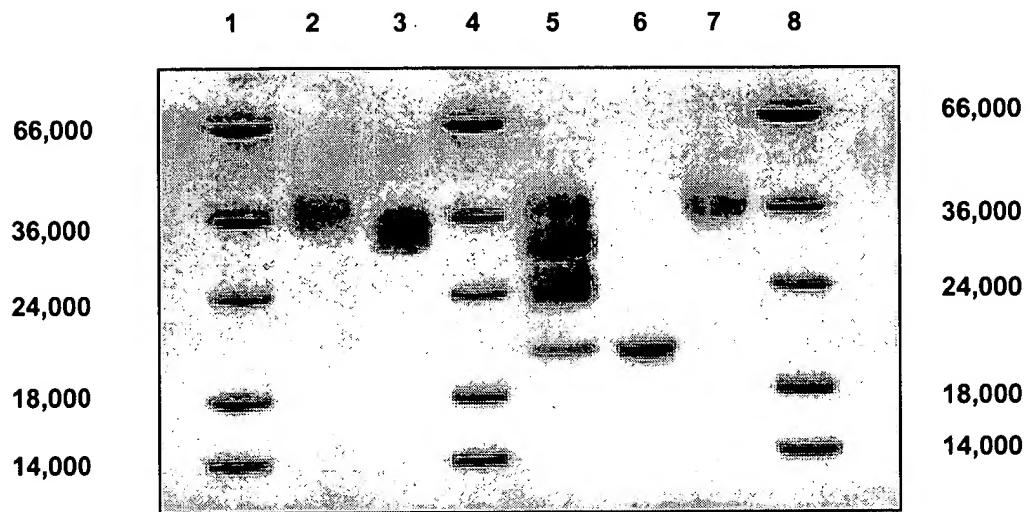
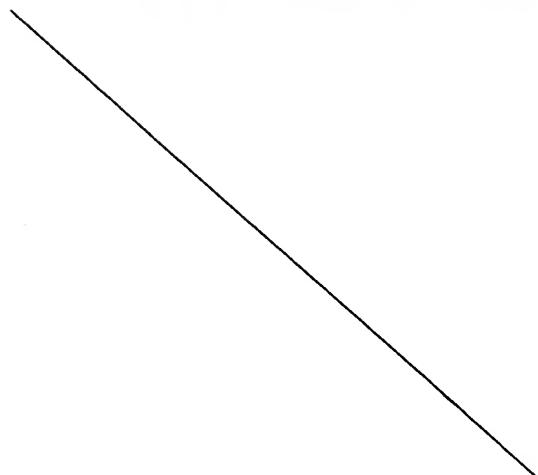
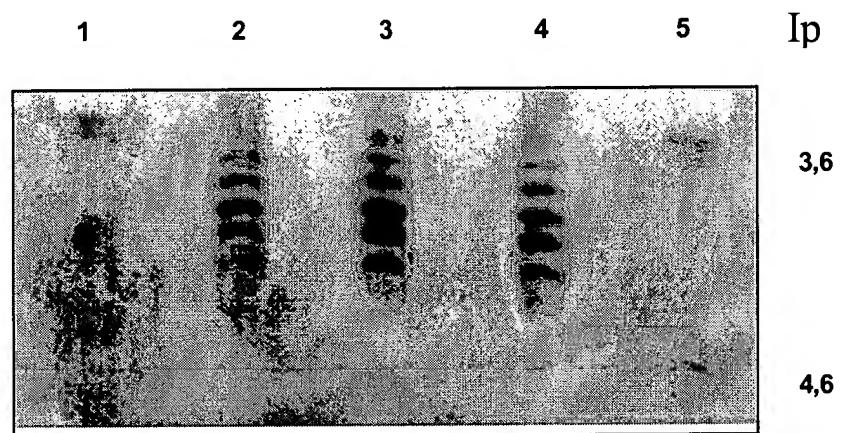


Fig. 4. Determination of Isoelectric Point (Isoelectric focusing)-----





**VIII. Deposit of Microorganisms -----**

The pDHFR vector was deposited on February 2, 1999 in the Argentine Cell Bank Association (ASOCIACIÓN BANCO ARGENTINO DE CÉLULAS – ABAC) under access code ABAC-P002.

The pVex 1 vector was deposited on February 2, 1999 in the Argentine Cell Bank Association (ASOCIACIÓN BANCO ARGENTINO DE CÉLULAS – ABAC) under access code ABAC-P001.

The claimed recombinant cell line was deposited on February 2, 1999 in the Argentine Cell Bank Association (ASOCIACIÓN BANCO ARGENTINO DE CÉLULAS – ABAC) under access code ABAC-L-200.

**IX. Abstract -----**

The gene encoding for human erythropoietin (EPO) was obtained from human genomic DNA. The gene used does not include fragments from neighboring regions to the 5' and 3' of the EPO gene that do not encode for the protein. The gene was cloned in an expression plasmid for eukaryotic cells that has as unique expression control elements the early promoter of the SV40 virus and its polyadenylation signal. Recombinant cells resulting from transfection with the genetic constructs used, provide an unexpectedly high level of expression, i.e. 50 mg of recombinant EPO per liter of culture medium per day.

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**ASOCIACIÓN BANCO ARGENTINO DE CÉLULAS -Argentine Cell Bank Association-**-----

Legal Entity Nº: 1,530,806-----

Upon the signed request of Dr. Aída Sterin Prync (on behalf of Laboratorios Bio Sidus S.A.), it is hereby certified that, the biological material, whose names and codes are detailed below, is stored in liquid nitrogen at the Cell Cultures laboratories of the Instituto Nacional de Enfermedades Virales Humanas – National Institute of Human Viral Diseases- (Monteagudo 2510-2700 Pergamino, Argentina), under the custody of the Asociación Banco Argentino de Células - Argentine Cell Bank Association- (ABAC), from February 2, 1999.:-----

NAME-----CODE-----

pVEX PLASMID-----ABAC-P-001-----

PDHFR PLASMID-----ABAC-P-002-----

46M-----ABAC-L-200-----

Buenos Aires, February 2, 1999-----

There appears a signature.-----

Dr. Ana M. Ambrosio-----

-

There appears a seal that reads Asociación Banco Argentino de Células – Argentine Cell Bank Association-.-----

TECHNICAL DATASHEET----- (10) -----

PUBLICATION No.: AR-----

(21) APPLICATION No.:-----

On the left, there appears the Argentinian Coat of Arms -----

(19) -----

51) INT. CL:-----

I.N.P.I.-----

Argentine Republic-----

12) X INVENTION PATENT-----

UTILITY MODEL-----

(22) FILING DATE: -----

(30) PRIORITY DATA:-----

(41) APPLICATION PUBLICATION DATE: -----

BULLETIN No.:-----

(61) ADDITIONAL TO: -----

(62) DIVISIONAL FROM:-----

(71) APPLICANT: BIO SIDUS S.A.-----

Constitución 4234. Buenos Aires Argentina-----

(72) Inventor(s):-----

(74) Agent. License 611/900 -----

(83) Microorganisms deposit: Deposited in -ABAC- Asociación Banco Argentino de Células- Argentine Cell Bank Association – Access Code: ABAC-P002-----

(54) TITLE OF THE INVENTION: "Recombinant Human Erythropoietin Producing Cell Line and the Recombinant Human Erythropoietin produced by this cell."-----

(57) ABSTRACT: -----

The encoding gene for recombinant erythropoietin (EPO) was obtained from human genomic DNA. The gene used does not include fragments of 5' and 3' neighboring regions of the EPO gene that do not encode for the protein. The gene was cloned in a expression plasmid for eukaryotic cells which has the early promoter of SV40 virus and its polyadenylation signal, as unique elements of

expression control. The recombinant cells resulting from the transfection with the genetic constructions used, unexpectedly produced more than 50 mg of recombinant EPO per liter of culture media per day.-----

AR-----

Most representative diagram No.-----

There appears the Argentinian Coat of Arms-----

MINISTRY OF ECONOMY AND PUBLIC SERVICES-----

NATIONAL INSTITUTE OF INDUSTRIAL PROPERTY-----

INVENTION PATENT-----

February 23, 1999-----

Reception Desk-----

National Institute of Industrial Property-----

INVENTION PATENT.-----

Application of: Invention Patent-----File:-----

Under Proceeding P990100679, an INVENTION PATENT application has been filed-----

Date: 02/23/1999-----Time: 00:00-----

Resp: Coll Areco, Carlos Miguel-----P19990100679-----

File Bar Code-----Query Priority Order-----

01999010067923020000-----P19990100679-----

Argentine Republic. There appears the Argentinian Coat of Arms. I.N.P.I.-----

-  
APPLICATION OF-----

-  
INVENTION PATENT: X-----

MODEL OF UTILITY CERTIFICATE-----

FILING DATE: P679-02-23-99-----

There appears a seal that reads: Dr. Eduardo Arias. Legal Countersigner. Patent National Administration-----

Applicant -----

1) Name and Surname / Company's Name:-----

BIO SIDUS S.A.-----

2) Identity Document:-----

Marital Status:-----

Marriage:-----

Spouse's Name:-----

3) Retirement Account or Pension Funds Management (AFJP): CUIT-----

No of CUIL (Unique Labor Identification Code) or CUIT (Unique Tax Identification Code): 30-59811709-4. ----- V.A.T: Liable for V.A:T Registration.- -----

4) Registered in the National Industrial Registry (Decree-Law 19,971/72) No.- -----

5) Real Address: Constitución 4234. Buenos Aires. Argentina.-----Legal Address: Alsina 971 – 1º Piso, of.10, Buenos Aires .-----

II. PURPOSE. -----

6) Title of invention: Recombinant Human Erythropoietin producing cell line and the recombinant human erythropoietin produced by this cell-----

7) Type of patent: -----

a) Final: for a 20-year period-----

b) Additional to Patent No.: -----

8) Act 17,011. Priority Date: -----

Country:-----

No. -----

III. Attached Documents -----

9) Enclosures:: -----

-  
a) Fee receipt for the requested service -----

- b) Annexed Form in duplicate -----
- c) Cover in duplicate -----
- d) Descriptive Memory in duplicate -----
- e) Signed claims in duplicate -----
- f) 2 copies of the first claim -----
- g) Diagrams in triplicate -----
- h) Number of boards -----
- i) Reduced-scale copies-----
- j) Certified copy (Act 17,011)-----
- k) Assignment Document -----
- l) Draft drawings-----

IV Legal Entities -----

10) The corporation, represented by HUMBERTO MARIO DE PASQUALE-----  
who states under oath that he is the attorney having his powers in force and that  
the corporation is registered on Date: 10/07/1983 No. 7258. Page: --Book:--  
Volume: A-----

V. Power of Attorney-----

11) Power of Attorney registered in -----  
Registered in the INPI (National Institute of Industrial Property) under No.: Other  
Registry: ----No. -----

12) In this case, authorization is given to: CARLOS MIGUEL COLL ARECO  
and/or HUGO EDUARDO MARTINEZ LAHITOU-----  
to proceed in this matter until its finalization with faculties to sign documents,  
wave, if necessary, and request certificates.-----

13) Power of Attorney is attached-----

14) Retirement Account or / Pension Funds Management (AFJP) CONSOLIDAR  
CUIL or CUIT No.: CUIT 20-04991729-6 CUIT: 20-16821007-9-----  
Agent No. 611/900 -----

VI Statement -----

16) By virtue of Decree with no number dated June 7, 1901 (on Patentability in foreign countries) it is declared that the invention has not been patented abroad.-

VII. Remarks: It is stated that the legalized copies of the corresponding Power of Attorney, Minutes of Incorporation, Minutes of General Meetings and of Board of Directors are attached to the invention patent application No. P980105609-----  
There appears a signature. Carlos Miguel Coll Areco (Signature of the authorized person). -----

There appears a signature. Humberto Mario De Pasquale. Attorney. (Applicant's Signature). -----

I, VIVIANA BEATRIZ CUMBO, A SWORN PUBLIC TRANSLATOR, DO HEREBY CERTIFY THE FOREGOING TO BE A TRUE TRANSLATION INTO ENGLISH OF THE PHOTOCOPY OF THE ORIGINAL DOCUMENT IN SPANISH LANGUAGE, ATTACHED HERETO, WHICH I HAVE HAD BEFORE ME. DONE AND SIGNED IN BUENOS AIRES, ON THIS FOURTEENTH DAY OF NOVEMBER, TWO THOUSAND AND THREE.-----  
-----

YO, VIVIANA BEATRIZ CUMBO, TRADUCTORA PÚBLICA MATRICULADA, CERTIFICO POR LA PRESENTE, QUE ÉSTA ES TRADUCCIÓN FIEL AL IDIOMA INGLÉS DE LA FOTOCOPIA DEL DOCUMENTO ORIGINAL REDACTADO EN IDIOMA CASTELLANO, ADJUNTA A LA PRESENTE, QUE HE TENIDO A LA VISTA, Y A LA CUAL ME REMITO, EN BUENOS AIRES, A LOS CATORCE DÍAS DEL MES DE NOVIEMBRE DE DOS MIL TRES.-----